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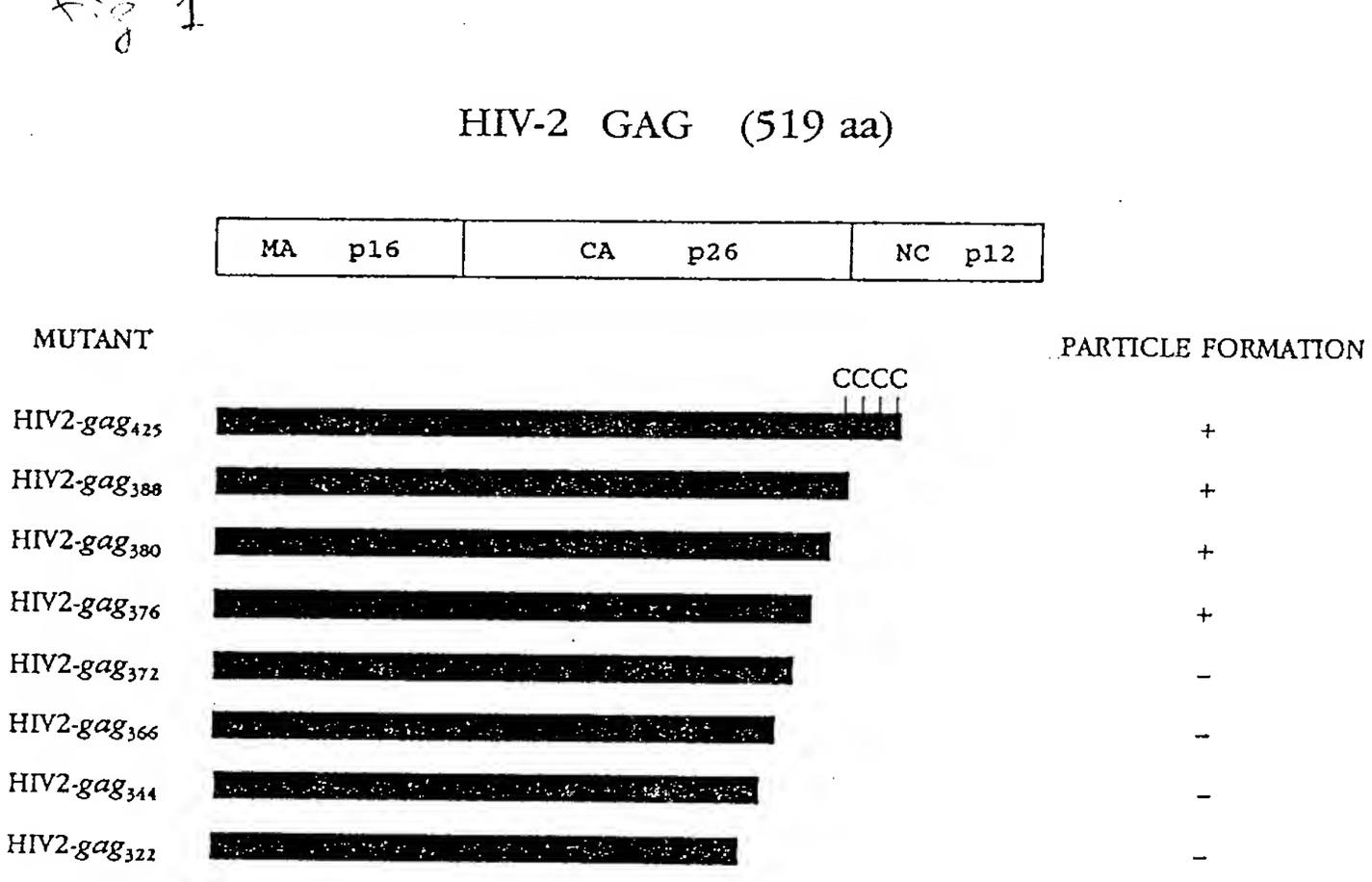
(54) **Design, construction and expression of chimeric proteins for development of vaccines and diagnostic reagents.**

(57) Chimeric proteins, and the vaccine comprising the chimeric protein are disclosed. The preparation comprises linking *gag* of HIV to *env* to form the chimeric gene, inserting the obtained chimeric gene into the DNA of baculovirus, infecting insect cells or insect, with the resultant recombinant virus culturing it and purifying the obtained chimeric protein. The *gag* chimeric protein of HIV according to the present invention retains both antigenic and immunogenic properties.

Fig 1



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BACKGROUND OF THE INVENTION

1. Field of the Invention

The present invention relates to construction of chimeric proteins useful for AIDS vaccine, for development of diagnostic reagents and a process for production thereof. More particularly, the present invention relates to *gag* chimeric protein of HIV expressed in the recombinant baculovirus infected insect cells, and a process for production thereof.

2. Description of the Prior Art

Type 1 and 2 of the human immunodeficiency viruses(HIV) are recognized as the etiologic agents for acquired immunodeficiency syndrome(AIDS). The vaccine against the said viruses is an ideal way of preventing the said syndrome from infection with HIV, therefore much researches have been focused on molecular biological analyses of structures and functions of HIV. The main virion structural proteins of HIV are derived from the three structural genes known as *gag, pol, env.* The genome of many different isolates of HIVs have been completely sequenced and amino acids sequences have been deduced from the cloned proviral DNA sequences. The envelope gene of HIV codes for a glycoprotein precursor with of molecular weight 160,000(gp160). The precursor gp160 in virus infected cells is processed(or cleaved) to produce envelope glycoprotein gp120 and gp41. The envelope glycoprotein gp120 of HIV has been the major target for developing a candidate vaccine against AIDS. gp120 recognizes the cellular receptor(CD4) on helper T lymphocytes and carries the V3 loop domain that induces neutralizing antibodies(Science 234, 1392-1395, 1986; Proc. Natl. Acad. Science, USA 83, 7023-7027).

The V3 loop represents the third hypervariable region of HIV-1 gp120(amino acid residues 308-331) which contains not only a major immunodominant neutralizing epitope but also the epitopes for antigen-dependent cellular cytotoxicity(ADCC) and cytotoxic T-lymphocyte(CTL) recognition. Although the majority of the amino acids in the V3 loop are variable among different strains of HIV, a G-P-G-R motif at the tip of the loop is conserved(Science 249, 932-935, 1990).

Recently, Huang et al. and Björling et al. demonstrated that the principal neutralization domain of the envelope glycoprotein of HIV-2 is also located in the region corresponding to the hypervariable motif in the V3 loop of HIV-1 gp120. The CD4-binding region, which is located within C-terminal third of HIV-1 gp120-(amino acid residues 397-439) plays an essential role in infectivity of HIV. This region also seems to be weakly immunogenic because it forms a pocket which is not accessible to immune system, thus high-titre neutralizing antibody against this region is not presently available.

SUMMARY OF THE INVENTION

gag−env chimeric proteins can be obtained by linking *gag* of HIV to env to form the chemeric gene, inserting the obtained chimeric gene into the DNA of baculovirus, infecting the resulted recommbinant virus with the insect cells or insect, culturing it and purifying the obtained chemeric protein. Such chimeric proteins is useful for AIDS vaccine.

BRIEF DESCRIPTION OF THE DRAWINGS

The invention will be described in detail with reference to the accompanying drawings in which :

Fig. 1 shows deletion mutants of HIV-2 *gag* gene and particle formation. A series of deletion mutants of HIV-2 gag gene were constructed by PCR with specific primers and the size of the deletion mutant are indicated by the amino acid numbers which are retained. Deletion mutants of HIV-2-gag_{425}, -gag_{388},-gag_{380}, and -gag_{376} formed virus-like particles and released into culture medium whereas deletion mutants HIV1-gag_{372}, -gag_{366}, -gag_{344}, -gag_{322} did not. Polypeptide domains of HIV-2 *gag* protein, p16 MA(matrix protein), p26 CA(capsid protein) and p12 NC(nucleic acid binding protein) are shown on the top of diagram. C on HIV-gag_{425} denoted cysteine which represent the zinc finger domain.

Fig. 2 shows site-specific mutants of HIV-2-gag_{380-W} gene with particle formation, Three prolines located at amino acid position 373, 375 and 377 at the C-terminus of *gag* mutant, HIV-2-gag_{380-W}, was substituted with 1,2 or 3 leucine residues by site-directed mutagenesis using PCR and expressed in SF9 cells. The subsitution of up to two prolines did not affect the *gag* particle formation, whereas changing of all three prolines resulted in the failing of particle formation as examined by electron microscopy.

Fig. 3 shows construction of chimeric *gag−env* genes. *Bg1*II fragments containing the V3(amino acid positions 273-363) or V3 + CD4BD(amino acid position 294-491) from HIV-2 gp120 were amplified by PCR. The V3 + CD4BD sequences of HIV-1 was directly isolated from pUC-gp120-NSS by *Bg1*II digestion. To insert these *Bg1*II fragments at the 3'-terminus of the HIV-2 gag gene without the protease sequences, two *Bg1*II sites were created by crossover linker mutagenesis, one downstream of the *Pst*I site and another at the C-terminus just upstream of the stop codon, *Bg1*II fragments carrying either V3 or V3 + CD4BD from HIV-1 or HIV-2 were inserted into either the middle of the 3'terminal *Bg1*II site. A total of six constructs were made. The dot-hatched box and solid black box represent the V3 and CD4BD of HIV-1 gp120, respectively. The checkered box and the box with vertical lines represent the V3 and V3 + CD4BD of HIV-2 gp120 respectively. The number of amino acids presented in each fragment is indicated.

Fig. 4 shows expression of chimeric *gag−env* proteins in SF9 cells infected by recombinant baculoviruses. Recombinant baculovirus infected SF9 cell lysates were analyzed by SDS-PAGE and proteins were stained with Coomassie blue(A) or detected by Western blots with pooled sera from AIDS pationts(B), Lanes 1-4: recombinant viruses AcNPV-HIV-2*gag*, Ac-*gag*M-1V3, Ac-*gag*C-1V3, and Ac-*gag*C-1V3 + 1CD4. The chimeric *gag−env* particles released into the culture supernatant were purified by centrifugation in 20-60% discontinuous sucrese density gradients, subjected to SDS-PAGE and detected by Coomassie blue staining(C) and Western blot(D). Lane 1: Purified *gag* particle; Lanes 2-4 : Purified *ga*gC-1V3, *gag*C-2V3 and *gag*C-2V3 + 2CD4 chimeric particles; M, Marker proteins; C, uninfected cell control; Wt, wild-type AcNPV-infected cells. The major fusion protein P55 and P66 are indicated with arrows and possible cleavage products are indicated by open arrows.

Fig. 5 shows electron micrographs of sucrose gradient-purified *gag−env* chimeric particles. (A) HIV-2 *gag* particles produced by SF9 cells infected with recombinant AcNPV-HIV-2 *gag*, (B) Chimeric *gag−env* particles produced by recombinant Ac-*gag*C-1V3. (C) Chimeric *gag* particles produced by recombinant Ac-*gag*C-2V3, and (D) Chimeric *gag* particles produced by recombinant Ac-*gag*C-2V3 + 2CD4. Samples were stained with uranyl acetate. The bar represents 100 nm.

Fig. 6 shows immunoblot analysis of chimeric *gag−env* proteins. The chimeric *gag−env* proteins and gp120 of HIV-1 and HIV-2 were subjected to SDS-PAGE and electro-transferred to nitrocellulose filters. Filters were incubated with rabbit antisera specific for HIV-1 gp120 (a) and HIV-2 (B) and with ^{125}I-labeled protein A. Lane 1: HIV-2 *gag* protein; lane 2, gp120 protein; lane 3, chimeric *gag*C-1V3 protein; lane 2, gp120 protein; lane 3, chimeric *gag*C-1V3 protein; lane 4, chimeric *gag*C-1V3 + 1CD4 protein; C, cell control; W, wild type AcNPV-infected cell control. The rabbit antisera against HIV-1 and HIV-2 gp120 have been described elsewhere.

Fig. 7 Western blot analysis using rabbit antisera made against chimeric *gag−env* particles. (A) Anti-*gag*C-1V3 serum recognized non-glycosylated gp120 protein of HIV-1. Lane 1, HIV-2 *gag* protein; Lane 2, non-glycosylated gp120 protein of HIV-1. (B) Anti-*gag*C-2V3 serum recognized non-glycosylated gp120 protein of HIV-2. Lane 1, HIV-2 *gag* protein; lane 2, non-glycosylated gp120 protein of HIV-2. Wt, wild-type AcNPV-infected cells at day 3 postinfection(p.i.). C, cell control. Sera were diluted 1:200 and the Bio-Rad immuno-Blot AK detection system was employed.

Fig. 8 shows neutralization of HIV-1_{IIIB} and HIV-2_{ROD} infection with immune rabbit sera. Antisera against *gag*C-1V3 and *gag*C-2V3(HIV-2) chimeric particles were diluted and tested for neutralization of virus using reverse transcriptase and viral p24 or p 26 assays. a and b: HIV-1; and d: HIV-2. Pre-immune serum(▮) ; V3 specific immune serum (▒) from rabbits immunized with chimeric *gag*-V3 particles of HIV-1 or HIV-2; anti-gp120 sera(▒) specific for gp120 of HIV-1 OR HIV-2. The neutralizing activity of anti-*gag*C-1V3 and anti-*gag*C-2V3 sera were determined by incubation of sera(1:5 dilution) with stock virus preparation of HIV-1_{IIIB}(5000 $TCID_{50}$) or HIV-2_{ROD} (8000 $TCID_{50}$) at 37 °C for 1 hour before infecting H9 cell. Viral infection was monitored by reverse transcriptase activity(a and c) and the production of HIV-1 p24(b) or HIV-2 p26(d) *gag* proteins at 1-16 days p.i.

Other objects, features and advantages of the invention will be hereinafter become more readily apparent fromthe following description.

DETAINLED DESCRIPTION OF THE PREFERRED EMBODIMENTS

The present invention provides the *gag* chimeric protein of HIV, which retain both antigenic and immunogenic properties of *gag* and a portion of *env* proteins.

The present inventors reported previously that expression of the *gag* coding sequences of HIV-2, lacking the protease gene, in insect cells produced virus-like particles(Virology 179, 874-880, 1990).

The C-terminus of the gag protein, including the zinc finger domain, is not necessary for particle formation(Fig. 1). Therefore, it is possible to replace the C-terminus of the *gag* precursor protein with other sequences without losing the ability of the gag protein to form virus-like particles. The *gag* protein has the unique ability to form particles in the absence of all other components of the virus, and the chimeric *gag* particles are devoid of genomic RNA. Formation of chimeric *gag* particles containing the major neutralizing epitope(V3) and/or the CD4-binding domain(CD4BD) of gp120 may allow efficient generation of HIV-neutralizing antibodies: antigens presented in a particulate form may enhance immunogenicity of the epitopes and multiple copies of specific epitopes can be presented. Furthermore, secreted chimeric particles can be safely and easily collected and purified from cell culture media by centrifugation.

In this application, we show mapping of essential domains of *gag* protein for particle formation and construction of six different chimeric *gag* genes containing either the V3 loop(V3) or the V3 loop plus the CD4 binding domain(V3 + CD4BD) of gp120 from HIV-1 or HIV-2. These constructs were expressed in insect cells using a baculovirus expression vector. The minimum length of HIV-2 gag to form particles is 376 amino acids and 373rd position proline is essential for the particle formation. The chimeric gene construction reveals that only certain combinations of fusion proteins are expressed, assembled as virus-like particles, and retain antigenicity and immunogenicity of both *gag* and *env* epitopes.

The present invention provides a map of the minimum length of HIV-2 *gag* gene sequences which are required for the gag particle formations using deletion mutagenesis.

The amino acids which is deleted up to 143 amino acids at the C-terminus from the 519 amino acid sequence, which retains 376 amino acids at the N-terminus is the minimum protein for the gag particle formation of HIV-2. In contrast, deletion of four more amino acids, which retain 372 amino acids at the N-terminus, abolished the particle formation.

Site-directed mutagenesis revealed that the proline at amino acid position 373rd, but not 375th and 377th, is essential for the particle formation.

To map the *gag* protein domain(s) which is essential for the particle formation, both deletion and site-directed mutagenesis were carried out. Fig. 1 clearly demonstrates that 376 amino acids at N-terminus are required for the particle formation. In contrast to previous reports, the zinc-finger domain at the C-terminus is not required for the gag particle formation, Furthermore, the present site-specific mutants showed that the 373rd position proline is essential for the particle formation as shown in Fig. 2. The results in Fig. 1 and Fig. 2 indicate that one must retain uninterrupted 1,128 base paris of HIV-2 gag reading frame is essential to package foreign epitopes into *gag* particles.

The present inventors have constructed six different combinations of chimeric genes by coupling the truncated HIV-2 *gag* gene which will code for 425 amino acids to the neutralizing domain(V3) or neutralizing and the CD4 binding domains(V3 + CD4BD) of gp 120 *env* gene sequences from HIV-1 or HIV-2. Such a preparation has led to the completion of the present invention.

The *env* gene sequences were either inserted into the middle of the *gag* gene or at the 3' terminus of the *gag* gene. Virus-like particles were formed by chimeric gene products only when the env gene sequences were linked to the 3' terminus of the *gag* gene. Insertion of *env* gene sequence in the middle of the *gag* gene resulted in high level chimeric gene expression but without the formation of virus-like particles.

Especially, three different chimeric proteins: (1) *gag*(425 amino acids) with HIV-1 V3(91 amino acids), (2) *gag* with HIV-2 V3(90 amino acids) and (3) *gag* with HIV-2 V3 + CD4BD(198 amino acids) formed virus-like particles that were secreted into the cell culture medium(cf. Table 1).

TABLE 1

Comparison of particle formation and yield of chimeric fusion proteins			
Recombinant Viruses	Particle formation*	Sucrose concentration**	Yield/5x10^8 cell /liter
AcNPV-HIV-2*gag*	+	40%	30mg
Ac-*gag*M-1V3	-	-	-
Ac-*gag*C-1V3	+	50%	6mg
Ac-*gag*M-2V3	-	-	-
Ac-*gag*C-2V-3	+	50%	25mg
Ac-*gag*C-2V3 + 2CD4BD	+	60%	2mg

note) * Plus sign denotes virus-like particles were recovered from the cell culture media.
** Virus-like particles were banded on top of these sucrose solutions after ultracentrifugation.

Accordingly, the present invention provides a recombinant baculovirus containing chimeric gene.

Recombinant baculovirus of the present invention can be prepared by using *Autographa Californica nuclear polyhedrosis* virus(AcNPV) or *Bombyx iridexcent* virus.

The recombinant virus is obtained by inserting the chimeric gene into AcNPV genome and purified by the consecutive plaque assays to select polyhedrin negative recombinant virus The AcNPV inserted with gag of HIV-2 is deposited with the ATCC as a deposit number VR2314, AcNPV inserted with gag of HIV-2 and V3 of HIV-1 as a number VR2316, AcNPV inserted with gag of HIV-2 and V3 of HIV-2 as a number VR2317, on February 26, 1991 respectively. These are guaranteed for furnishing for the purpose of research.

The insect cells selected from the group consisted of *Spodoptera frugiperda* cells, *Mamestra brassica* cells, *Trichoplusia ni* cells, *Bombyx mori* cells and *Bombyx mori* silkworm cells were infected with recombinant baculovirus obtained as above to make expression of *gag* chimeric protein.

The chimeric *gag* particles of the invention elicit neutralizing antibodies in the rabbits which completely block HIV infection.

The chimeric *gag* particles of the invention can be used for the antigen of AIDS vaccine or of diagnostic reagent. The AIDS vaccine according to the present invention exhibits specific immune reaction against immunogen including vaccine used at pre or post exposure for prevention from infection of HIV, or used for immunotherapeutic treatment.

The vaccine of the invention may include the conventional absorbent, stabilizer, adjuvant, carrier etc. and it may be administered through the conventional route.

Other features of the invention will become apparent in the course of the following description of the exemplary embodiments which are given for illustration of the invention and are not intended to be limiting thereof.

EXAMPLES

Example 1 〈Preparation of plasmids〉

Autographa californica nuclear polyhedrosis virus(AcNPV) and recombinant AcNPV were grown and assayed in *Spodoptera frugiperda*(SF9) cell monolayers using complete TNM-FH medium containing 10% fetal bovine serum at 27 °C as described in Adv. Virus Res. 35, 177-192, 1988. Wild type AcNPV DNA was purified by the method of Smith and Summers(Virology 89, 517-527, 1978).

Plasmids pHXB-2D and p1BM containing the entire HIV-1_{HXB2D} and HIV-2_{NIHZ} genomes respectively were obtained from Dr. R. Gallo(National Institutes of Health, Bethesda, MD). The recombinant plasmids pUC19-gp120-NSS, pUC18-gp120 and pUC19-GAG containing full-length cDNA copies of the HIV-1 *env*, HIV-2 *env* and a truncated *gag* gene of HIV-2(which codes 425 amino acids) respectively were subcloned from pHXB-2D and p1BM as described by the inventors in Virology 179, 874-880, 1990.

Example 2 〈Preparation of chimeric gene〉

To subclone the V3 and V3 + CD4B of the HIV-1 and HIV-2 *env* genes into pUC19-GAG recombinant plasmid pUC19-gp120-NSS and pUC19-gp120 were used as a templates and the specific regions were

amplified with appropriate oligonucleotide primers. That is to say, plasmids pUC19-gp120-NSS(HIV-1) and pUC18-gp120(HIV-2) were used as templates for polymerase chain reaction(PCR) amplification of an HIV-1 DNA fragment corresponding to V3 domain(91 amino acids, position 273-363) and HIV-2 DNA fragments corresponding to V3 equivalent domain(90 amino acids, position 294-383) and V3 + CD4BD(19 amino acids, position 294-491). All primers were designed to create a *Bg1*II restriction site at the 5' end so that gp120 DNA fragments could be inserted into the *Bg1*II site of pUC19-GAG.

The HIV-1 V3 DNA fragment was amplified using L-1: (5'-CGAAGATCTGTCAATTTCACGG-3') and L-2-(5'-GCAGATCTTTGCTTAAAGATTA-3') primers, which are complementary to nucleotides 816 through 836 and 1075 through 1089 respectively. The V3 + CD4BD DNA fragment of HIV-1 was generated from pUC19-gp120-NSS *Bg1*II digestion at nucleotide positions 816 and 1398 of gp120. Primers L-3(5'-CGAGATCTCATTGTTAAGAGGCC-3') and L-4 (5'-GCAGATCTTCTGCAGTTAGTCC-3'), complementary to nucleotides position 879 through 893 and 1135 through 1149 of HIV-2 gp 120 respectively, were used to synthesize the HIV-2 V3 DNA fragment. Primers L-3 and L-5 (5'-GCAGATCTCTTTACTGATGTAG-3'), which is complementary to nucleotides 1459 through 1473 of HIV-2 gp120 were used to synthesize the HIV-2 V3 + CD4BD DNA fragment. PCR was performed according to the procedures provided with the Geneamp kit(Norwalk, CT). Briefly, 20 ng of linearized gp120 DNA was added to 100 μl PCR reaction mixture(10mM Tris-HCl, 50mM KCl, 1.5 mM $MgCl_2$ and 0.01% gelatin) containing 20 μM each dNTP, 2.5 Units of Taq polymerase, and 20 μM of each oligonucleotide primer.

The V3 and V3 + CD4BD genes were amplified for 30 PCR cycles(94 °C for 1 min., 45 °C for 3 min.). The chimeric *gag* gene constructs containing the V3 and V3 + CD4BD fragments were confirmed by dideoxy DNA sequencing of double stranded DNA, using the Sequence Kit(trademark of United States Biochemical Corporation).

*gag*M-1V3, *gag*C-1V3, *gag*C-1V3 + 1CD4BD, *gag*M-2V3, *gag*C-2V3, *gag*C-2V3 + 2CD4BD chimeric genes were prepared by the above method. The numbers 1 and 2 in front of V3 and CD4BD denote genes from HIV-1 and HIV-2 respectively, gagM denotes the chimeric gene inserted into the middle of the *gag* gene, and *gag*C denotes the chimeric gene inserted into the terminus of the *gag* gene. The procedure for generation of the present chimeric gene showed briefly in Fig. 3.

Example 3 〈Production of Recombinant Baculovirus〉

The chimeric genes obtained from the Example 3 were isolated after digestion with *Bam*HI and inserted into the baculovirus transfer vector pAcYM1. SF9 cells were co-transfected with mixtures of infectious wild type AcNPV DNA(1 μg) and recombinant plasmid DNAs(4 μg) using the standard procedure(Adv. Virus Res., 35, 177-192, 1988).

Among the recombinant baculoviruses obtained from the above procedure, AcNPV carrying *gag* of HIV-2(hereinafter called 'AcHIV2GAGYK')was deposited as a deposit number VR2314, AcNPV carrying *gag* of HIV-2 and V3 of HIV-1 (hereinafter called 'AcHIV2GAG + 1V3YK') as a deposit number VR2316, and AcNPV carrying *gag* of HIV-2 and V3 of HIV-2(hereinafter called 'AcHIV2GAG + 2V3YK') as a deposit number VR2317 with the American Type Culture Collection(ATCC) on February 26, 1991 and these are guaranteed for furnishing of samples for the purpose of research.

After incubation of recombinant baculovirus at 27 °C for 4 days, culture supernatants were harvested and titrated on 80% confluent monolayers of SF9 cells. To obtain polyhedrin-negative recombinant AcNPV, the plaques lacking polyhedra were picked, purified by three consecutive plaque assays, and used to produce virus stock of $2x10^8$ PFU/ml,

Example 4 〈Expression of Chimeric particles〉

SF9 cells were infected with either wild type AcNPV or recombinant AcNPV carrying chimeric genes at multiplicities of infection of 5 PFU/cell and incubated at 27 °C. After appropriate incubation times(usually 3 to 4 days), cells were harvested and washed twice with PBS. Whole cell lysates were prepared by resuspending the cell pellet in water and adding an equal volume of 2x dissociation buffer(10% beta-mercaptoethanol, 10% SDS, 25% glycerol, 100 mM Tril-HCl, pH 7.0, 0.04% bromophenol blue). Cell lysates were analyzed by electrophoresis in 12% polyacrylamide gel containing SDS(SDS-PAGE) and the protein bands were visualized by staining with Coomassie blue(Nature 227, 283-303, 1987).

Western blot analyses were then performed using sera from AIDS patients and the Bio-Rad Immune Blot AK system. The results showed in Fig. 4.

Fig.4A shows the proteins produced by recombinant baculoviruses Ac-*gag*M-1V3, Ac-*gag*C-1V3 and Ac-*gag*C-1V3 + 1CD4BD. Since the original HIV-2 *gag* protein has 93 amino acids deleted from the C-

terminus, an insertion of V3(91 amino acids) or V3 + CD4BD(194 amino acids) of HIV-1 gp120 into the *gag* gene would be expected to produce proteins of 55 KDa and 66 KDa, respectively.

As shown in Fig.4A, strongly stained protein bands migrating at either 55 KDa or 66 KDa were observed in the lysates of SF9 cells infected with Ac-*gag*M-1V3(lane 2), Ac-*gag*C-1V3(lane 3) and AC-*gag*C-1V3 + 1CD4BD(lane 4) but were not present in lysates of mock or wild type baculovirus-infected cells.

Western blot analysis revealed that both the p55 and p66 fusion proteins were recognized by pooled HIV-1 positive human sera(Fig.4B, lane 2, 3, and 4). No specific reaction was observed with mock- and wild type AcNPV-infected cells.

The results clearly demonstrate that insertion of either HIV-1 V3 or V3 + CD4BD into the *gag* protein resulted in expression of proteins at levels at least as high as that of recombinant AcNPV-HIV-2*gag* alone-(Fig.4A, lane 1).

Similar results were obtained when three other recombinant baculoviruses containing the V3 and V3 + CD4BD genes from HIV-2 gp120, Ac-*gag*M-2V3, Ac-*gag*C-2V3, and Ac-*gag*C-2V3 + 2CD4BD were analyzed.

Example 5 〈Purification of Chimeric particles〉

The cell culture fluids obtained from the Example 5 was collected after centrifugation at 1,000 x g for 20 min. Chimeric particles in the culture supernatant were collected by ultracentrifugation in a Beckman SW 28 rotor at 80,000 x g for 1 hr and resuspended in PBS containing 0.1% Tween 20, 10 μg/ml aprotinin and stored at 4 °C. A band containing *gag* particle was collected from a 20-60% discontinuous sucrose gradient, diluted at least 10-fold with PBS and pelleted in an SW28 rotor at 80,000 x g for 1 hour. The pellet was gently suspended in PBS. *gag*C-1V3 and *gag*C-2V3 particles were recovered from the 50% sucrose cushion, whereas *gag*C-2V3 + 2CD4BD chimeric particles banded on top of the 60% sucrose cushion. The results of the morphology of sucrose gradient purified particles by transmission electron microscopy using uranyl acetate staining was shown in Fig. 5.

HIV-2 gag particle were spherical (Fig.5A), had diameters of approximately 100 nm, and were similar to those of mature HIV-1 particles budding from HIV-1 infected cells. The chimeric particles *gag*C-1V3(Fig. 5B), *gag*C-2V3(Fig. 5C) and *gag*C-2V3 + 2CD4BD(Fig. 5D) exhibited morphologies similar to the *gag* particles. The peripheral granular material frequently showed striations with a periodicity suggesting a helical arrangement. It formed a shell-like layer probably inside a lipid membrane and was presumably rigid. The only difference between the *gag* particles and chimeric *gag* particles was that the chimeric *gag* particles are slightly larger with approximate diameters of 130 nm, similar to the diameter of mature HIV-1 particles.

Experimental Example 1 〈Antigenity of Chimeric *gag* Particles〉

Antigenicity of chimeric *gag* particles was investigated by immunoblot analysis. Purified chimeric *gag* particles were subjected to SDS-PAGE and analyzed by Western blotting with rabbit antisera directed against HIV-1 and HIV-2 gp120 and ^{125}I-labeled protein A. The results are shown in Fig. 6. In this Fig. 6, lane 1 is HIV-2 *gag* protein; lane 2, gp120 protein; lane 3, chimeric *gag*C-1V3 protein; lane 4, chimeric *gag*C-1V3 + 1CD4 protein; C, cell control; and W is wild type AcNPV-infected cell control.

The HIV-2 *gag* protein was not recognized by anti-gp120 sera(Fig. 6A and B, lane 1) while the nonglycosylated forms of gp120 of HIV-1 and HIV-2 showed strong reactivity with their corresponding antisera(Fig.6A and B, lane 2). The 55 KDa and 66 KDa fusion proteins were specifically recognized by rabbit antisera against HIV-1 or HIV-2 gp120s(Fig.6A and B, lanes 3 and 4). The results clearly demonstrate that the chimeric proteins can be detected by antiserum specific for gp120 and reaffirm that inserted sequences of V3 or V3 + CD4BD are antigenic.

Experimental Example 2 〈Immunogenicity of Chimeric gag-V3 Particles〉

In order to examine the capacity of particles to induce antibodies to both *gag* and *env* proteins of HIV, rabbits were immunized four times at 4 week intervals with purified *gag*C-1V3 and *gag*C-2V3 chimeric particles. The immune rabbit sera were collected 2 weeks after the last immunization and tested for their ability to recognize gp120 of HIV-1 and HIV-2.

As shown in Fig. 7, the antisera made against both *gag*C-1V3 and *gag*C-2V3 chimeric particles recognize not only carrier HIV-2 *gag* protein(Fig.7A, B, lanes 1) but also non-glycosylated gp 120 of HIV-1-(Fig. 7A, lane 2) and HIV-2(Fig.7B, lane 2).

In contrast, neither wild type AcNPV infected SF9 cells nor uninfected SF9 cells contain proteins which were recognized by the antisera. These results clearly demonstrate that the V3 loop domain in chimeric gagC-1V3 and gagC-2V3 particles retain both antigenic and immunogenic properties.

Experimental Example 3 〈Immune Sera Against Chimeric *gag*-V3 Particles of HIV-1 or HIV-2 Neutralize Virus Infectivity in Vitro〉

Rabbit anti-sera directed agaginst chimeric particles were used to neutralize the infectivity of HIV as assayed by reverse transcriptase(RT) activity and *gag* p24 production. Rabbits were immunized four times at one month intervals with intramuscular injections of 25 μg of the density gradient-purified chimeric *gag* particles. Rabbit anti-*gag*C-1V3 and anti-*gag*C-2V3 sera(25 μl) were mixed with 100 μl of virus which represented 5000 $TCID_{50}$ of $HIV\text{-}1_{IIIB}$ or 8000 $TCID_{50}$ of $HIV\text{-}2_{ROD}$, respectively and the mixtures were used to infect H9 cells. The amount of p24 *gag* protein and reverse transcriptase(RT) activity in the culture media were assayed as quantitation of virus production on different days after infection (days 1-16) and the levels were compared with those of control samples in which virus was incubated with preimmune sera or rabbit anti-gp120 serum.

Fig. 8 shows that both rabbit anti-*gag*C-1V3 and anti-*gag*C-2V3 sera contained antibodies capable of neutralizing HIV infection of H9 cells. By day 9 postinfection, antisera to *gag*C-1V3 and *gag*C-2V3 chimeric particles completely blocked the production of HIV-1 and HIV-2, respectively. However, at day 12 post-infection, a small amount of HIV-1 was detected in cultures treated with anti-*gag*C-1V3 serum(Fig. 8, a and b). In contrast, the antisera to *gag*C-2V3 chimeric particles completely neutralized HIV-2 infectivity(Fig. 8, c and d). No reduction in RT and p24 *gag* protein production were observed with pre-immune sera. Rabbit anti-gp120 sera of HIV-2 showed stronger neutralizing activity of $HIV\text{-}1_{ROD}$ than rabbit anti-gp120 sera of HIV-1 against $HIV\text{-}1_{IIIB}$(Fig. 8).

From the above, it is recognized that the chimeric protein according to the present invention showed excellent neutralizing activity against HIV-1 and HIV-2.

Claims

1. A recombinant *gag−env* chimeric protein of HIV.

2. The *gag−env* chimeric protein according to Claim 1, in which *gag* is one of HIV-2.

3. The *gag−env* chimeric protein according to Claim 2, in which *gag* has at least 376 amino acids and not more than 425 amino acids from the N-terminus.

4. The *gag−env* chimeric protein according to Claim 3, in which *gag* forms the virus-like particles.

5. The *gag−env* chimeric protein according to Claim 4, in which the 373rd proline in the *gag* is essential for forming HIV virus-like particles.

6. The *gag−env* chimeric protein according to Claim 2, in which *env* is V3 loop of HIV gp120 or V3 + CD4BD of HIV-1.

7. The *gag−env* chimeric protein according to Claim 6, in which *env* is one of HIV-1 or HIV-2.

8. The *gag−env* chimeric protein according to Claim 1, in which *gag−env* chimeric protein is that expressed in insect cells using a recombinant baculovirus.

9. The *gag−env* chimeric protein according to Claim 8, in which *gag−env* protein is expressed in insect cells using a recombinant baculovirus selected from ATCC deposit number VR2316 and ATCC deposit number VR2317.

10. A recombinant baculovirus carrying the chimeric HIV *gag−env* genes in order to express the *gag−env* chimeric protein of HIV in insect cells.

11. The recombinant baculovirus according to Claim 10, in which the recombinant baculovirus is selected from ATCC deposit number VR2316 and ATCC deposit number VR2317.

12. A process for preparing HIV *gag−env* chimeric protein which comprises linking *gag* of HIV to *env* to form the chimeric gene, inserting the obtained chimeric gene into the DNA of baculovirus, infecting the resultant recombinant virus into insect cells or insect, culturing it and purifying the obtained chimeric protein.

13. A process for preparing HIV *gag−env* chimeric protein according to Claim 12, wherein the HIV gene is one coding for V3 loop of gp120 or V3 + CD4BD.

14. A process for preparing HIV *gag−env* chimeric protein according to Claim 13, wherein the V3 loop or V3 + CD4BD gene fragments are prepared by PCR method, and are inserted into plasmids.

15. A process for preparing HIV *gag−env* chimeric protein according to Claim 14, wherein primers for preparing the HIV-1 V3 fragments are selected from:

L-1 (5′-CGAAGATCTGTCAATTTCACGG-3′), and
L-2 (5′-GCAGATCTTTGCTTAAAGATTA-3′),
L-3 (5′-CGAGATCTCATTGTTAAGAGGCC-3′), and
L-4 (5′-GCAGATCTTCTGCAGTTAGTCC-3′),
L-3 (5′-CGAGATCTCATTGTTAAGAGGCC-3′), and
L-5 (5′-GCAGATCTCTTTACTGATGTAG-3′).

16. A process for preparing HIV *gag−env* chimeric protein according to Claim 12, wherein the baculovirus is *Autographa californica* nuclear polyhedrosis.

17. A process for preparing HIV *gag−env* chimeric protein according to Claim 12, wherein the insect cells are selected from the group consisting of *Spodoptera frugiperda* cells, *Mamestra brassica* cells, *Trichoplusia ni* cells, *Bombyx mori* cells and *Bombyx mori* silkworm cells.

18. A process for preparing HIV *gag−env* chimeric protein according to Claim 12, wherein the baculovirus is *Bombyx iridexcent* and the host cell is *Bombyx mori* silkworm cells.

19. Use of HIV-2 *gag* precursor protein as a foreign protein epitope carrier.

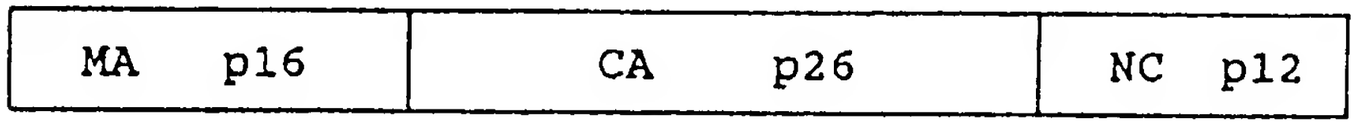
20. The maximum *env* gene sequence to form a chimeric *gag* particle is 198 amino acids attached to the C-terminus of *gag* gene which makes a total of 623 amino acids as hybrid protein.

21. A vaccine composition comprising *gag−env* chimeric protein of HIV with a pharmaceutically acceptable carrier therefor.

22. A vaccine composition according to Claim 21, in which the *gag−env* chimeric protein is one expressed from the recombinant baculovirus of ATCC VR 2316 or ATCC VR 2317.

Fig 1

HIV-2 GAG (519 aa)



MUTANT		PARTICLE FORMATION
	CCCC	
HIV2-gag_{425}		+
HIV2-gag_{388}		+
HIV2-gag_{380}		+
HIV2-gag_{376}		+
HIV2-gag_{372}		−
HIV2-gag_{366}		−
HIV2-gag_{344}		−
HIV2-gag_{322}		−

Fig 2.

SITE-SPECIFIC MUTAGENESIS

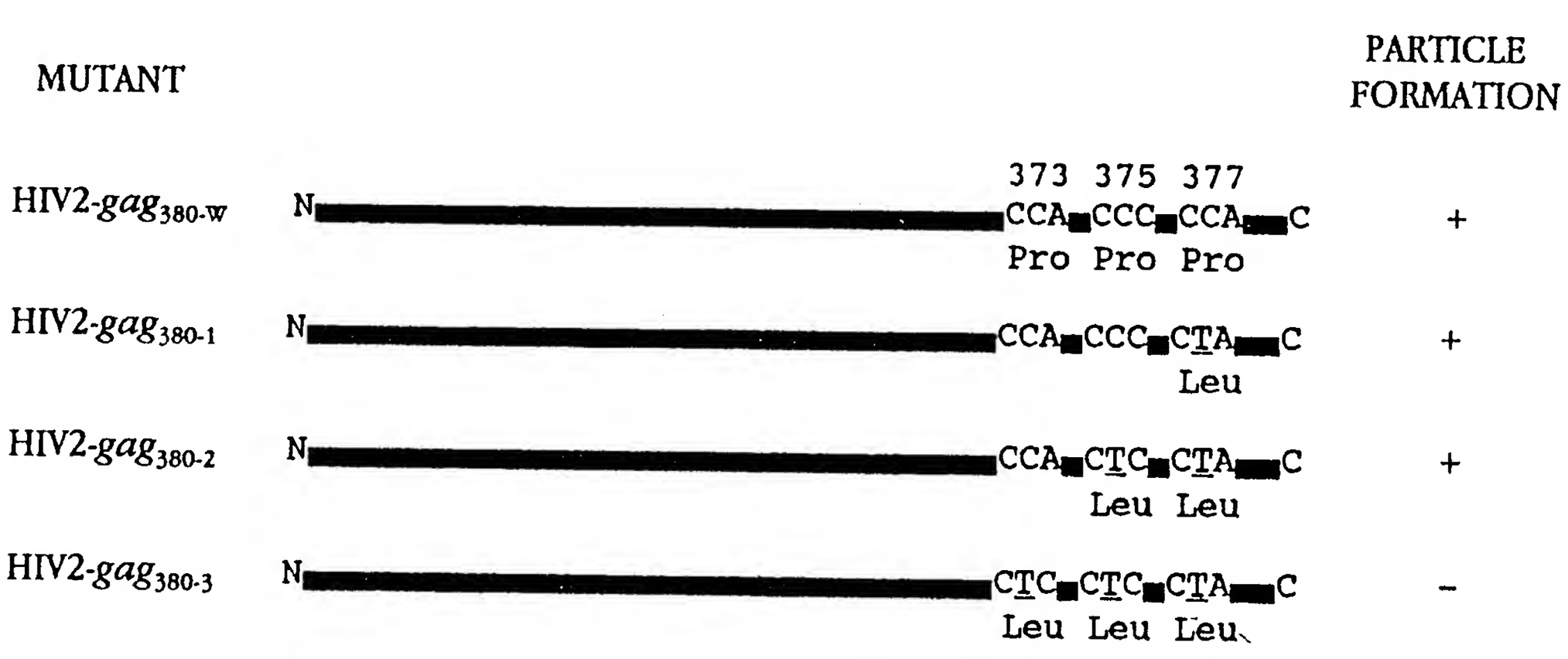


Fig. 3

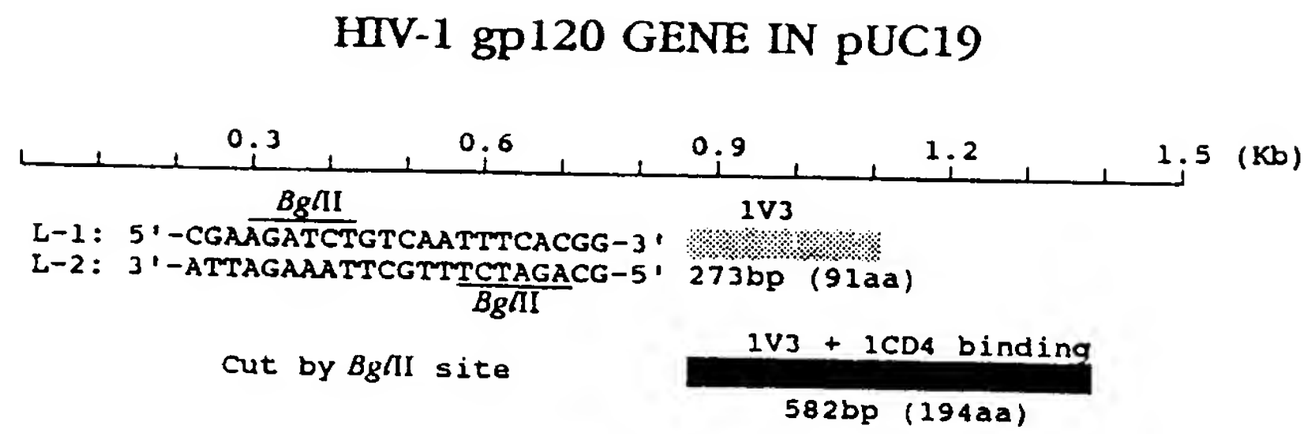


Figure 3

Fig 4

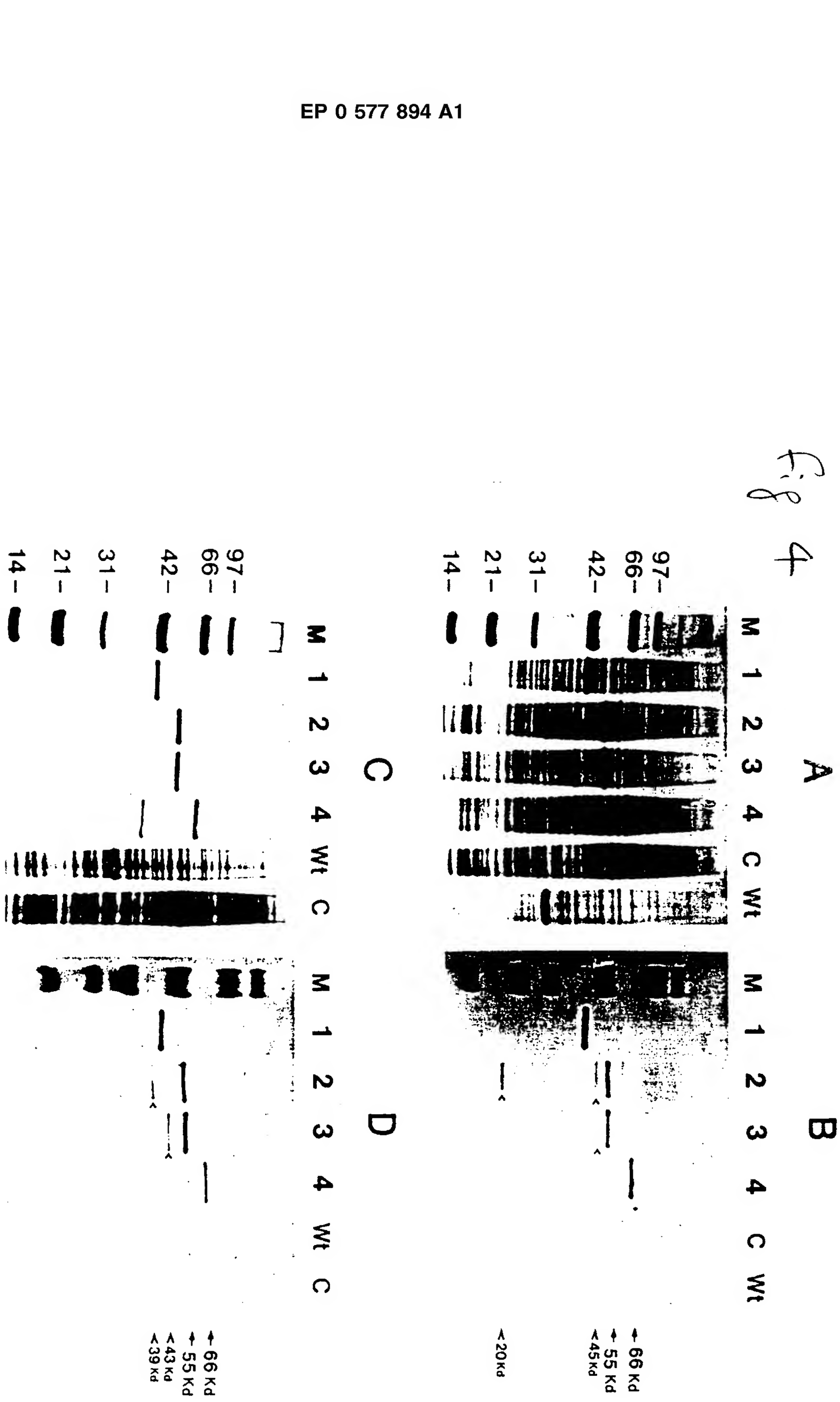


Figure 4

Figure 5

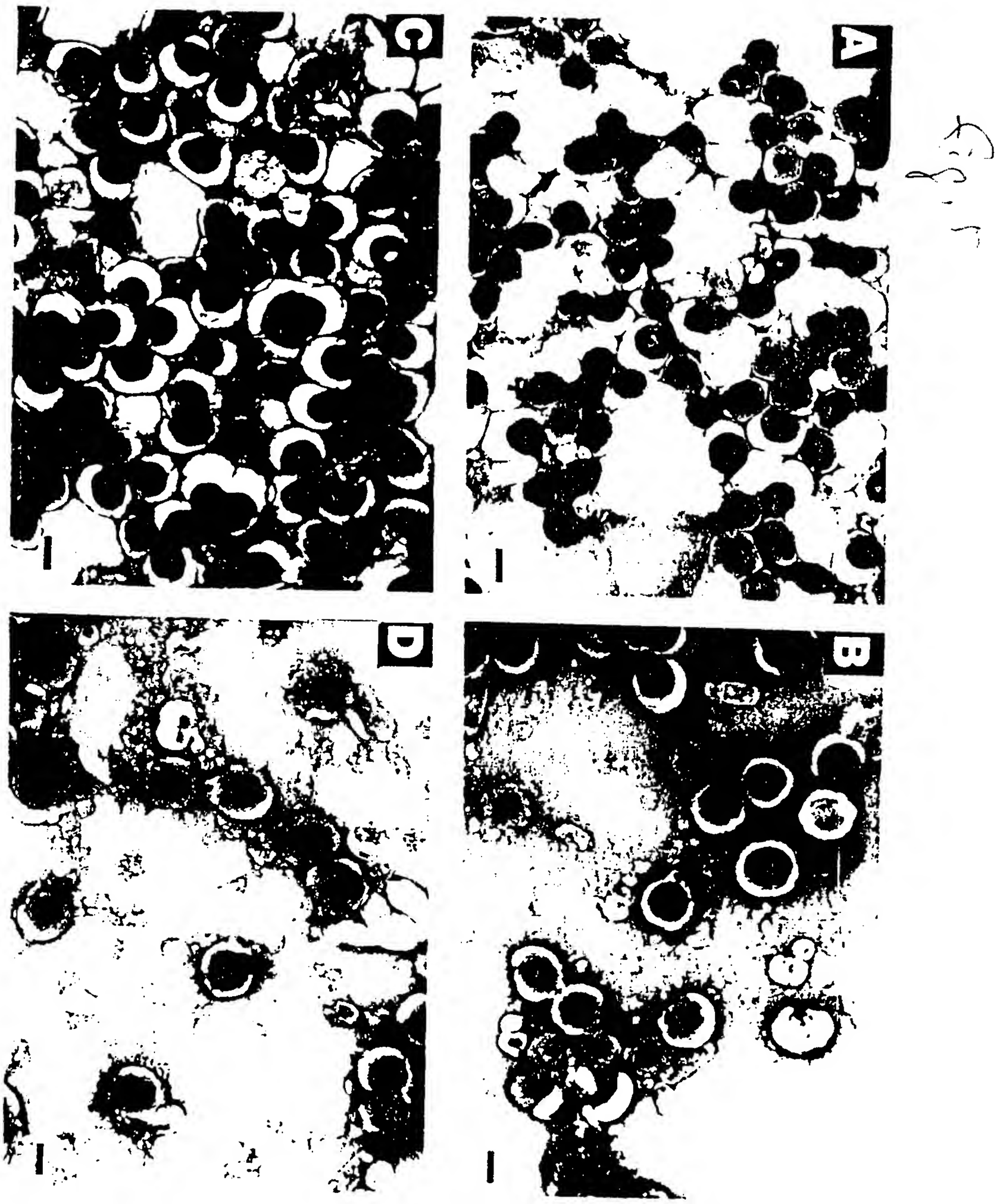


Fig. 6

A. Antiserum against HIV-1 gp120

B. Antiserum against HIV-2 gp120

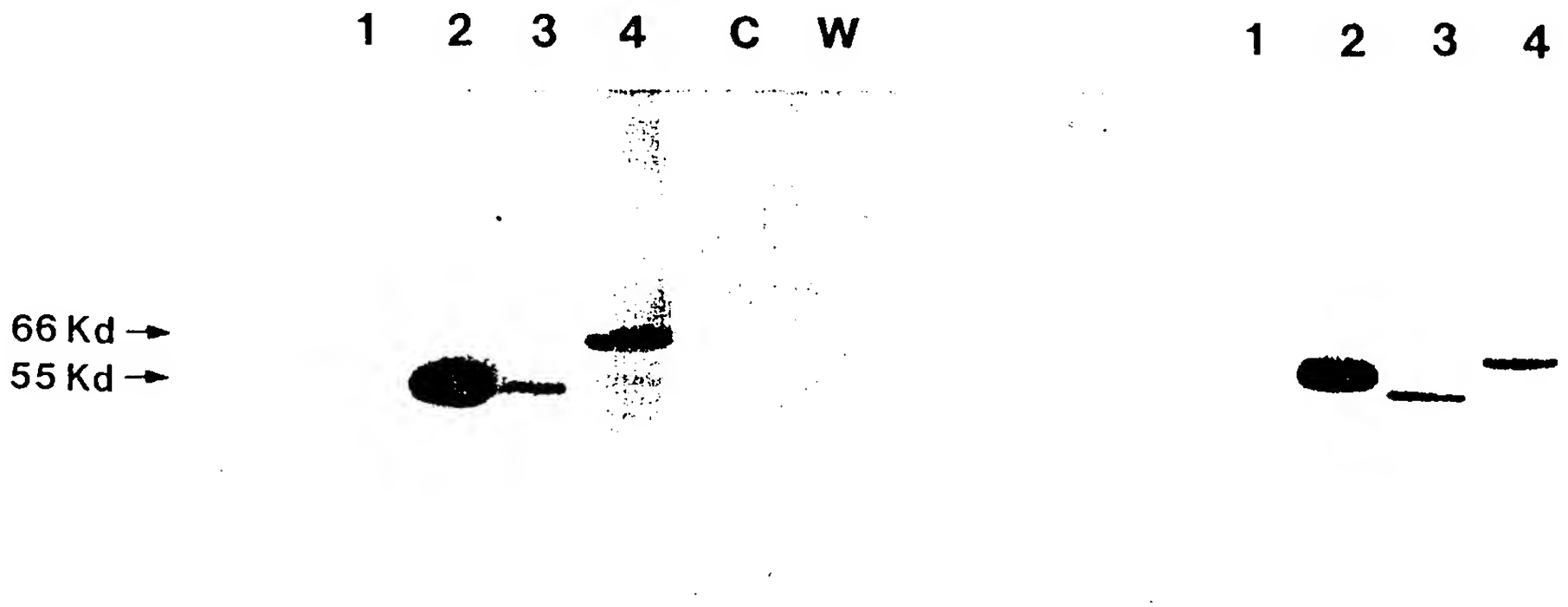


Figure 6

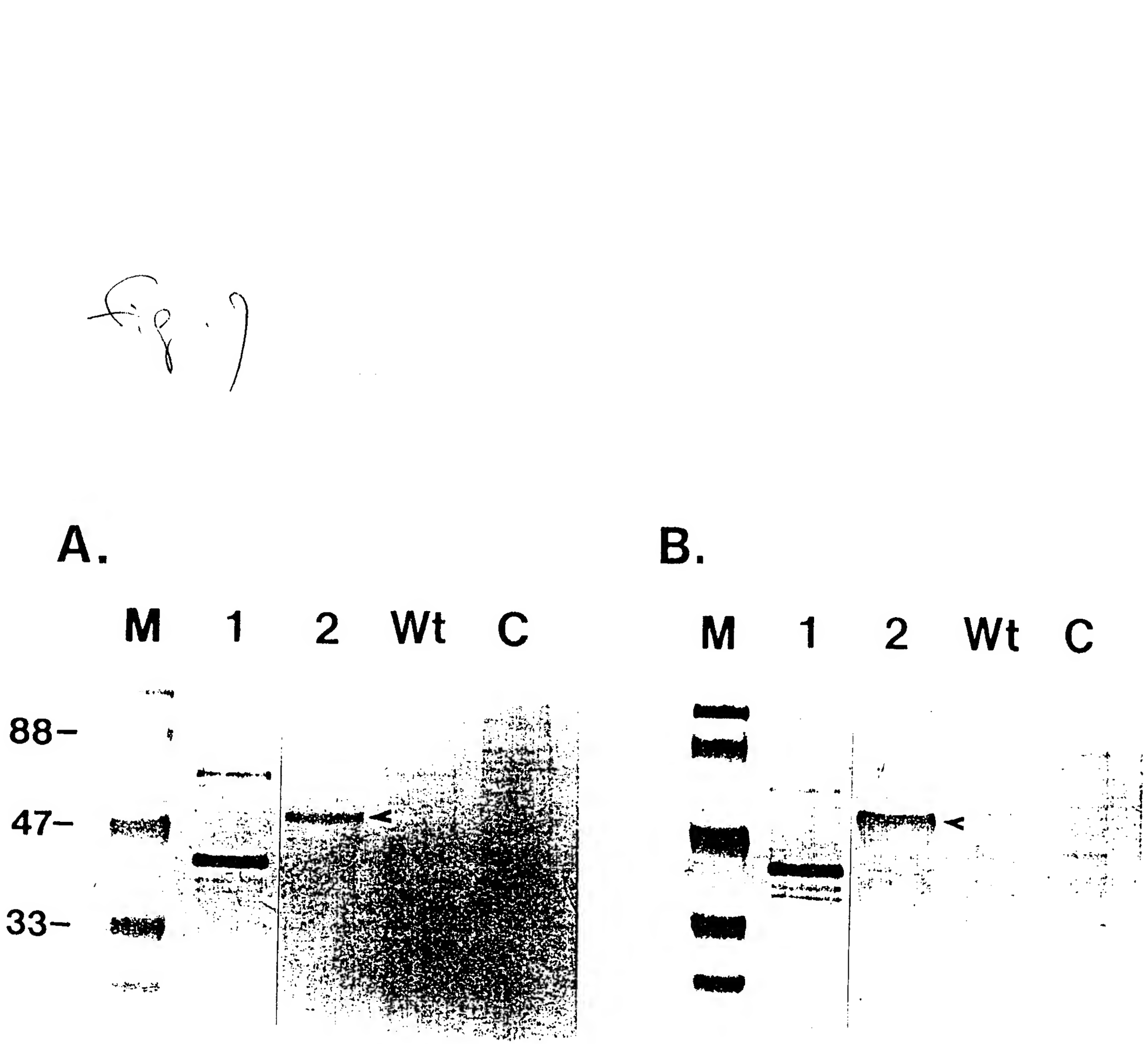
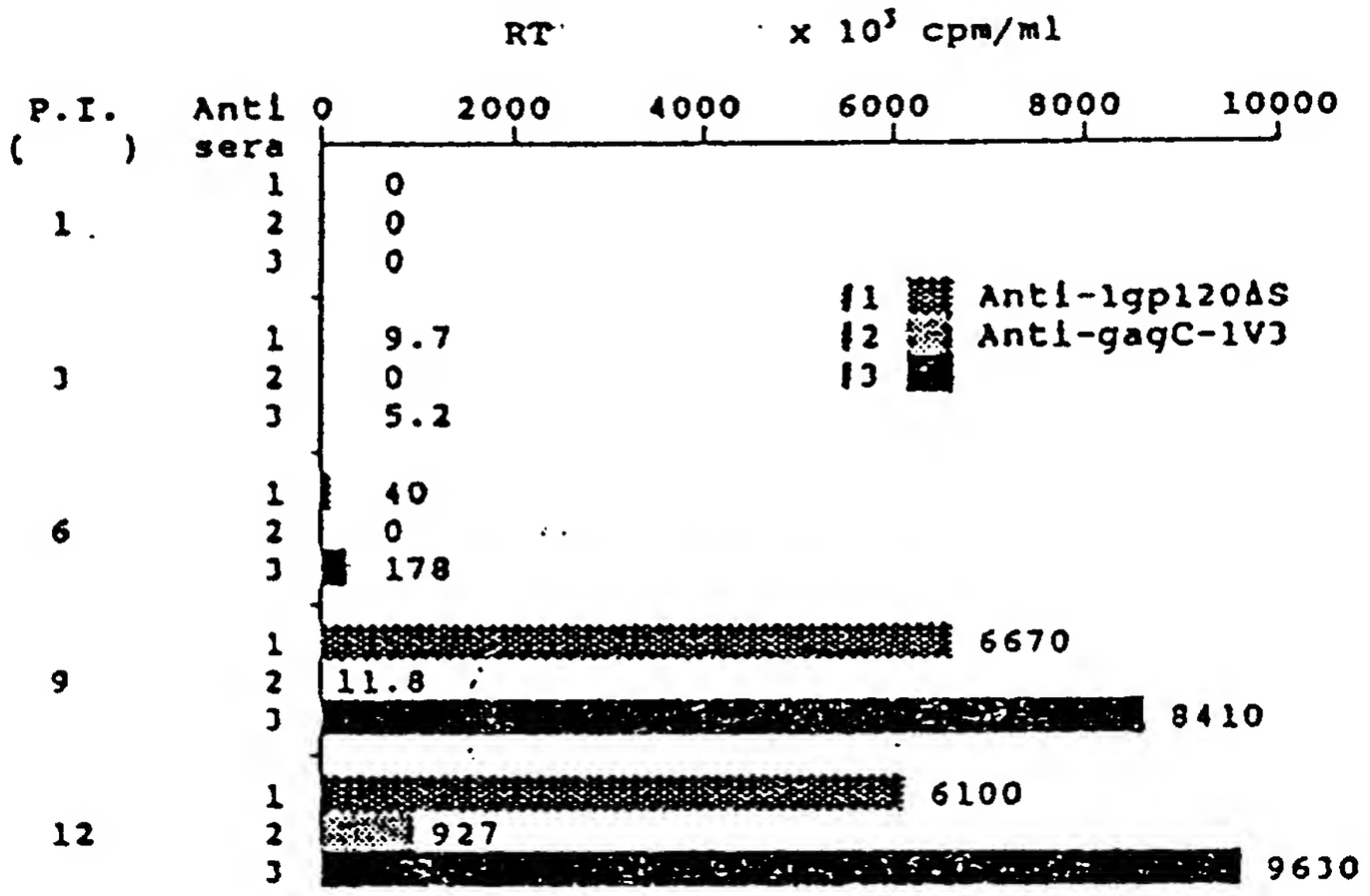


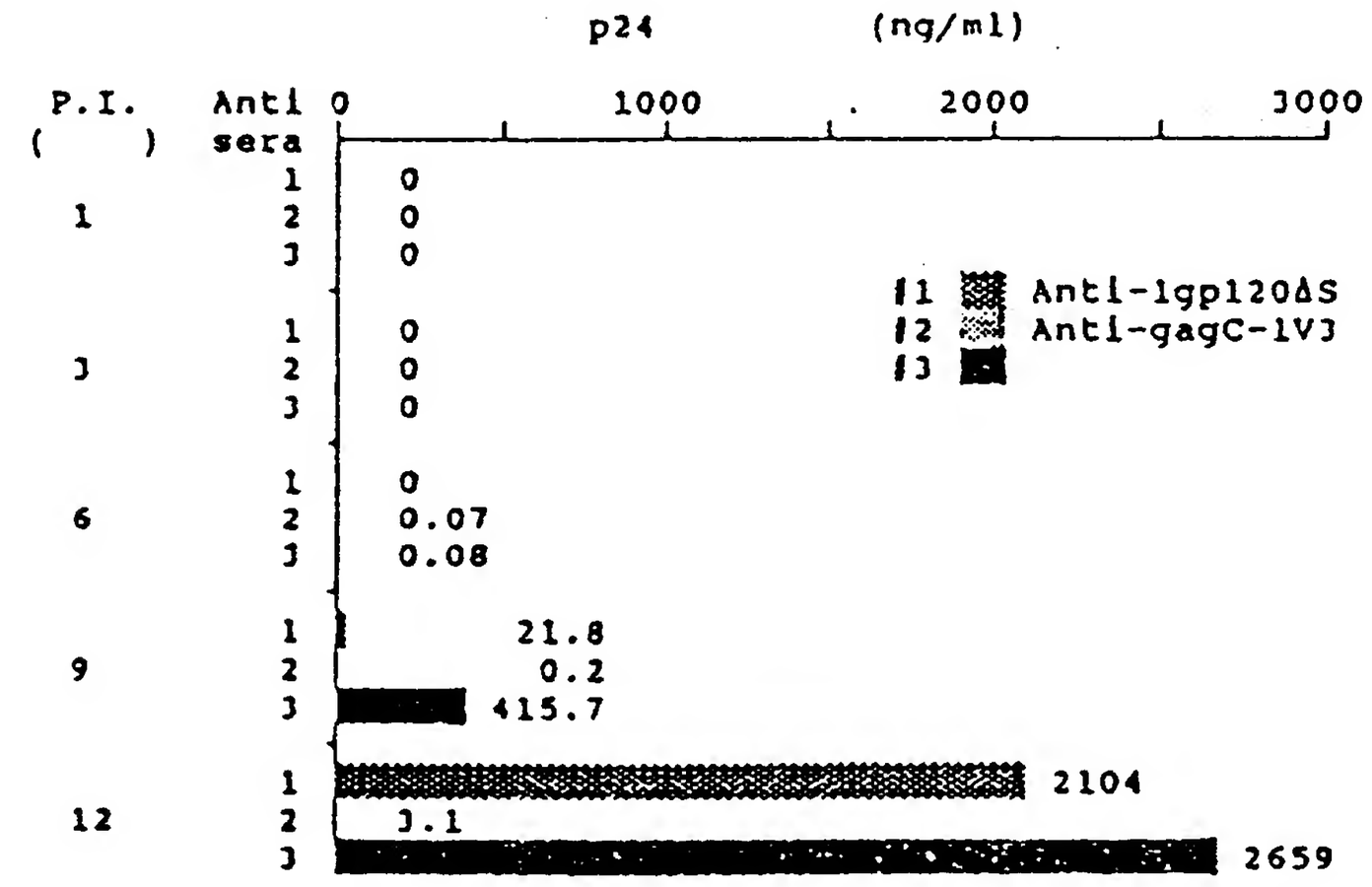
Figure 7

Fig. 8

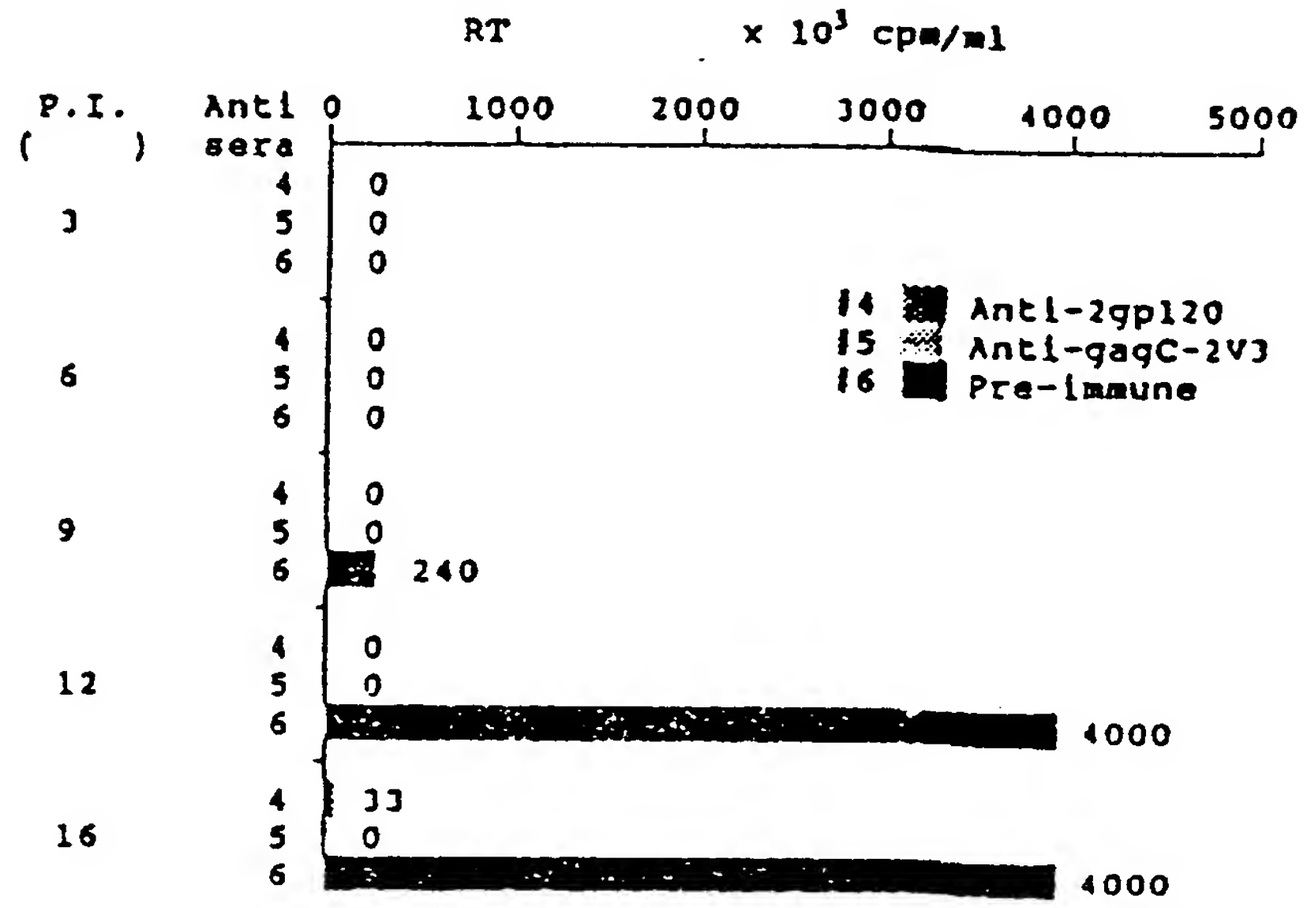
a. HIV-1



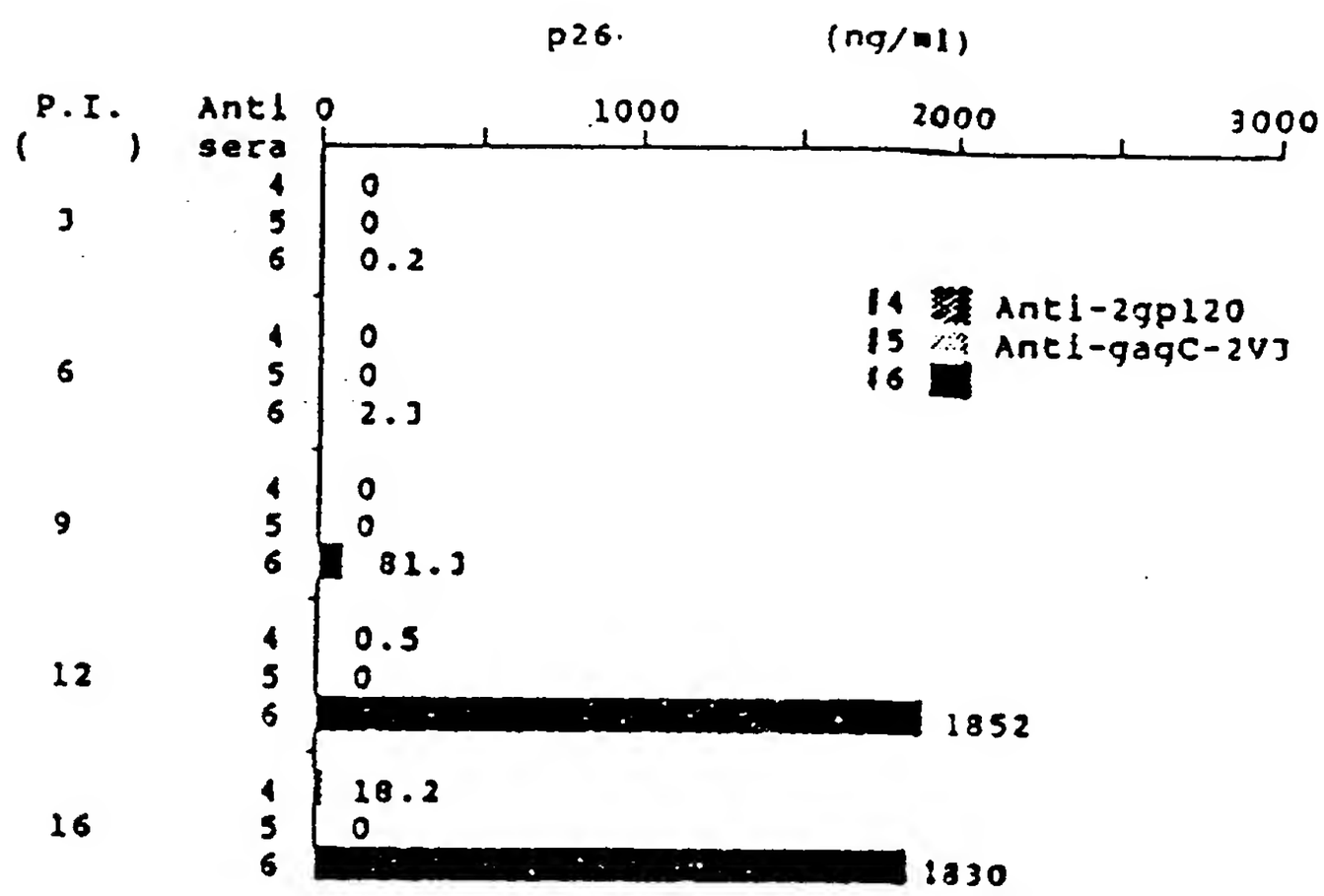
b. HIV-1 p24 gag



C. HIV-2



d. HIV-2 p26 gag



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EUROPEAN SEARCH REPORT

Application Number EP 92310032.5

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl.5)
P,X	CHEMICAL ABSTRACTS, vol. 118, no. 3, January 18, 1993, Columbus, Ohio, USA LUO, LIZHONG et al. "Chimeric gag-V3 virus-like particles of human immunodeficiency virus induce virus-neutralizing antibodies.", page 536, column 2, abstract-no. 20 650v & Proc. Natl. Acad. Sci. U.S.A. 1992, 89(21), 10527-31 --	1-22	C 07 K 15/04 C 12 N 7/01 C 12 N 15/48 A 61 K 39/21
A	CHEMICAL ABSTRACTS, vol. 116, no. 5, February 3, 1992, Columbus, Ohio, USA TRAUNECKER, ANDRE et al. "Bispecific single chain molecules (Janusins) target cytotoxic lymphocytes on HIV infected cells.", page 606, column 2, abstract-no. 39 564a & EMBO J. 1991, 10(12), 3655-9 --	1	
A	CHEMICAL ABSTRACTS, vol. 113, no. 15, October 8, 1990, Columbus, Ohio, USA WELDON, ROBERT A., Jr. et al. "Incorporation of chimeric gag protein into retroviral particles.", page 198, column 1, abstract-no. 127 642t & J. Virol, 1990, 64(9), 4169-79 --	12-14	
A	WO - A - 87/05 399	1	

TECHNICAL FIELDS SEARCHED (Int. Cl.5)
C 07 K 15/00 C 12 N 5/00 C 12 N 7/00 C 12 N 15/00 A 61 K 37/00 A 61 K 39/00 G 01 N 33/00

The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
VIENNA	23-09-1993	SCHARF

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Application Number

EP 92310032.5

DOCUMENTS CONSIDERED TO BE RELEVANT			
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl.5)
	(CENTOCOR INC.) * Claim 2 * ----		
			TECHNICAL FIELDS SEARCHED (Int. Cl.5)

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